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Inactivation of the *ybdD* Gene in *Lactococcus lactis* Increases the Amounts of Exported Proteins

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Random insertional mutagenesis performed on a *Lactococcus lactis* reporter strain led us to identify *L. lactis ybdD* as a protein-overproducing mutant. In different expression contexts, the *ybdD* mutant shows increased levels of exported proteins and therefore constitutes a new and attractive heterologous protein production host. This study also highlights the importance of unknown regulatory processes that play a role during protein secretion.

The food-grade Gram-positive bacterium *Lactococcus lactis* constitutes an interesting host for the production of heterologous secreted proteins to develop either nutraceuticals or new live-vaccine delivery systems (2, 5, 13, 19). However, yields are usually low. Production and secretion systems as well as engineered strains designed to increase the amounts and quality of secreted heterologous proteins have been established in *L. lactis*. This has been done by modifying either the secreted protein itself (11, 12) or the expression of host proteins that could be directly or indirectly involved in protein production (16, 21). We previously performed a random insertional mutagenesis in *L. lactis* strain MG *usp-nucT*, carrying a chromosome-inserted expression cassette that induces the production of UspNucT, a poorly secreted form of staphylococcal nuclease (NucT) fused to the signal peptide of the secreted protein Usp45 (25) (SP_{Usp}) used as a reporter to identify accessory genes encoding such host proteins. Nuc activity plate assay screening led to the isolation of mutants that were modified in their capacity to produce NucT (20). In this study, we focused on the *ybdD* mutant, in which NucT production was increased.

Spot tests performed on TBD agar plates identified three mutants with significantly higher nuclease activities than the control strain MG *usp-nucT*; all contained pGhost:IS*S1* integrations that mapped in the *ybdD* gene (Fig. 1A). We confirmed that this phenotype was not associated with pGhost9:IS*S1* insertion in the chromosome. After pGhost9:IS*S1* excision (15, 20), we compared the secretion profile of the *ybdD usp-nucT* (mutant I27; Fig. 1) mutant to the one obtained with the MG *usp-nucT* parental strain by Western blot analysis. Increased amounts of both intracellular precursor SP_{Usp}-NucT (pre-NucT) and secreted mature NucT were observed in the *ybdD* mutant (Fig. 1B). This result was confirmed by nuclease (Nuc) assay (23) (Fig. 1C).

In order to test the use of the *L. lactis ybdD* strain as a host for the improved production of heterologous proteins, we evaluated the effect of *ybdD* disruption on NucT production under the control of the NICE (for nisin-controlled expression) system (4). The plasmid pSEC:NucT carrying the expression cassette *usp-nucT* under the control of P_{nisA} (12) was introduced into two NZ9000 (9) derivatives: NZ(pIL253) (24), used as the negative control, and NZ*ybdD*, in which *ybdD* was inactivated by targeted insertional mutagenesis (14). Quantitative Western blot analysis of the NucT secretion profile obtained in these two strains shows a significant 2-fold increase of both nuclease forms in the *ybdD*-background strain (Fig. 2A and B), as confirmed by Nuc assay (Fig. 2C). This result demonstrates that the improvement associated with the *ybdD*-related phenotype is not restricted to heterologous protein expressed under the P_{usp} control but can also extend to heterologous protein production using another promoter as the NICE system.

As the effect of disrupting *ybdD* was analyzed only for the NucT reporter, an inefficiently secreted form of Nuc, fused to SP_{Usp}, the effect of the *ybdD* mutation was tested on the production/secretion profile of NucB, an efficiently produced and secreted form of Nuc in *L. lactis* that is cleaved extracellularly to give rise to the NucA form fused to an alternative efficient lactococcal SP, SP_{Exp4} (17, 22). The plasmid pLB141 carrying the expression cassette P_{nisA}-SP_{Exp4}:NucB (17) was introduced into the strains NZ*ybdD* and NZ(pIL253). Western blot analysis of the Nuc secretion profile in these two strains shows a significant 2-fold increase of both detected forms of nuclease in the *ybdD* context (Fig. 3A and B), as confirmed by Nuc assay (Fig. 3C). This result shows that the *ybdD* mutation is not restricted to an inefficiently produced and secreted heterologous protein and to a SP_{Usp45} secretion-driven protein. Moreover, production analysis of the major *L. lactis* secreted protein Usp45 (25) using Coomassie blue staining shows a similar ~2-fold increase in the amounts of Usp45 in the culture medium of the *ybdD usp-nucT* mutant compared to the wild-type strain (data not shown), showing that the *ybdD*-related phenotype is not Nuc specific.

Together, our results show that the *ybdD* mutant constitutes a very attractive host strain for achieving an improved yield of het-

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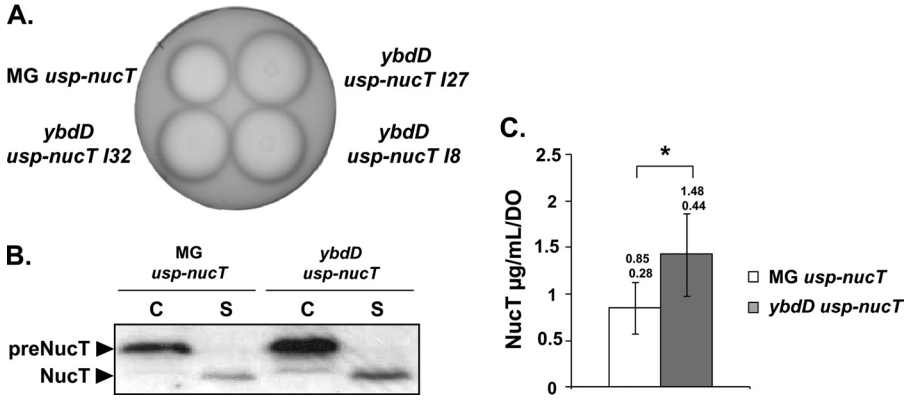

FIG 1 Isolation of the *L. lactis ybdD* overproducer mutant. (A) Nuclease activity plate assay of the three isolated *ybdD* mutants where pGhost9:IS*S1* (20) insertions occurred in the coding sequence of *ybdD* at nucleotide positions 495 (mutant I27), 597 (mutant I32), and 867 (mutant I8) with respect to the ATG start codon, allowing expression of a truncated form of YbdD leaving 54.2%, 65.4% and 95% of its N-terminal region. The same volumes of OD_{600}-adjusted stationary-growth-phase cultures of the three single *ybdD* mutants were spotted on TBD agar plates (10). (B) NucT secretion profile comparison obtained in MG *usp-nucT* and the *ybdD usp-nucT* mutant I27. Strains were grown exponentially, and protein samples present in cell (C) and supernatant (S) fractions were extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-Nuc antibodies. (C) Dosage of active Nuc released in the supernatant by MG *usp-nucT* and the *ybdD usp-nucT* mutant using a spectrophotometric nuclease assay (23). Data are means ± standard errors (SE) ($n = 8$). *, $P < 0.05$ (Mann-Whitney U test).

erologous secreted proteins in biotechnological applications. It would be of interest to analyze the effect of the *ybdD* mutation combined with numerous tools previously described as enhancers of production of heterologous secreted protein in *L. lactis* (17). Achieving a synergistic effect with the *ybdD* mutant would contribute to making *L. lactis* a more efficient cell factory for biotechnological applications.

As the *ybdD* effect was observed only on secreted proteins, we tested the production-improving *ybdD*-related phenotype on different cellular protein localizations by using either a cell-wall anchored form of the Nuc reporter or a cytoplasmic one. The plasmid pANC2608 carrying an expression cassette encoding the precursor SP_{Usp}:NucA:CWA_{M6} (pre-Nuc_{Anc}) under the transcriptional control of P_{59} was introduced into MG *usp-nucT* and the *ybdD usp-nucT* mutant, resulting in strain MG *usp-nucT*(pANC:NucA) and the *ybdD usp-nucT*(pANC:NucA) mutant. Two forms of cell wall-anchored Nuc were detected in the cell fraction: pre-Nuc_{Anc} and cell wall-anchored Nuc_{Anc} (Fig. 4A).

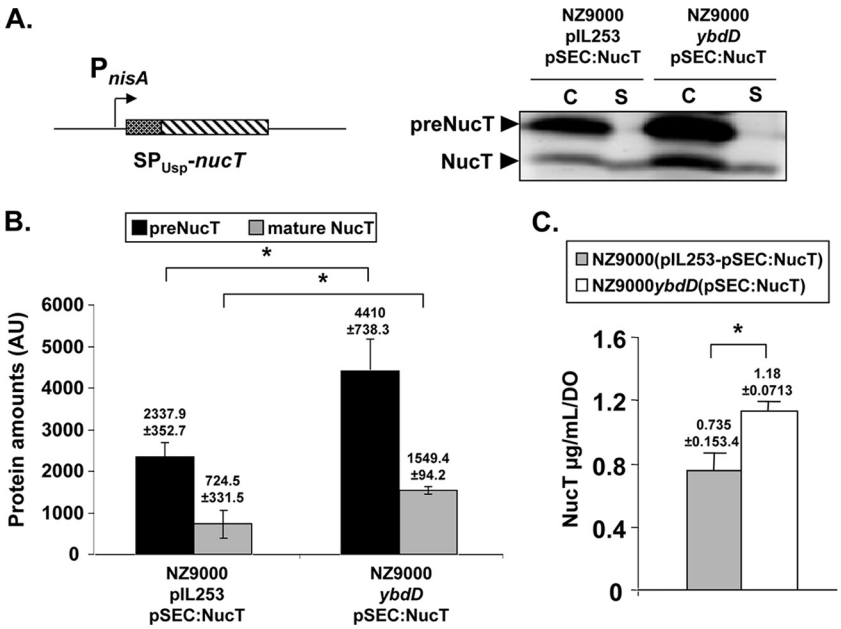

FIG 2 Effect of the *ybdD* mutation on NucT overexpression driven by the P_{nisA} inducible promoter. NucT production profiles of NZ(pIL253, pSEC:NucT) and NZ*ybdD*(pSEC:NucT) were compared. Protein samples from cell (C) and supernatant (S) fractions of exponentially growing cultures induced by 1 ng/ml nisin for 1 h were analyzed by SDS-PAGE and Western blotting using anti-Nuc antibodies (A) and quantified by fluorimetry (B); the total amount of each protein form (cell associated and released in the supernatant) is expressed in arbitrary units (AU) corresponding to the measured signal intensity. (C) Dosage of active nuclease released in the supernatant by NZ(pIL253, pSEC:NucT) and NZ*ybdD*(pSEC:NucT) strains using a spectrophotometric Nuc assay. Data are means ± SE ($n = 8$). *, $P < 0.05$ (Mann-Whitney U test).

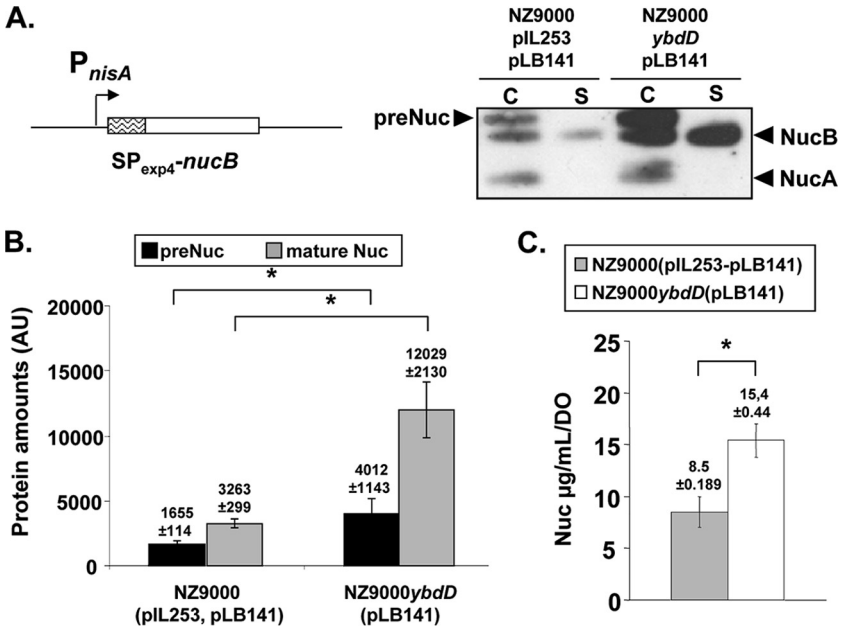

FIG 3 Effect of *ybdD* mutation on Nuc secretion driven by the SP_{Exp4} under the control of the inducible promoter P_{nisA}. NucB secretion profiles of NZ(pIL253, pLB141) and NZ*ybdD* (pLB141) are compared. Protein samples from cell (C) and supernatant (S) fractions of exponentially growing cultures induced by 1 ng/ml nisin during 1 h were analyzed by SDS-PAGE and Western blotting using anti-Nuc antibodies (A) and quantified by fluorimetry (B). Total amounts of the precursor form and both secreted forms (cell associated and released in the supernatant [NucB and NucA]) of the protein are expressed in arbitrary units (AU) corresponding to the measured signal intensity. (C) Dosage of active nuclease released in the supernatant by NZ(pIL253, pLB141) and NZ*ybdD*(pLB141) strains using a spectrophotometric Nuc assay. Data are means ± SE ($n = 6$). *, $P < 0.05$ (Mann-Whitney U test).

Compared to the control, both cell wall-anchored nuclease forms were detected in increased amounts in the *ybdD* mutant (Fig. 4A). This result shows that the production-improving phenotype of the *ybdD* mutant (i) is not restricted to secreted proteins and (ii) can increase the production of two different heterologous exported proteins in the same strain using two different promoters (Fig. 4). To determine whether the *ybdD* mutation could act on a cytoplasmic heterologous protein, the plasmid pCYT:Nuc carrying the expression cassette P_{nisA}-Nuc was introduced into NZ*ybdD* and NZ(pIL253), resulting in NZ*ybdD*(pCYT:NucB) and in NZ(pIL253-pCYT:NucB), respectively. Compared to the control strain, no differences were observed (Fig. 4B). Under P_{nisA} control, the *ybdD* mutation has an effect only on the exported forms of Nuc.

According to *L. lactis* IL1403 genome sequence annotation, *ybdD* encodes a hypothetical cytoplasmic protein of unknown function belonging to the Jag family of proteins (3). Sequence analysis of YbdD revealed the presence of KH and R3H domains,

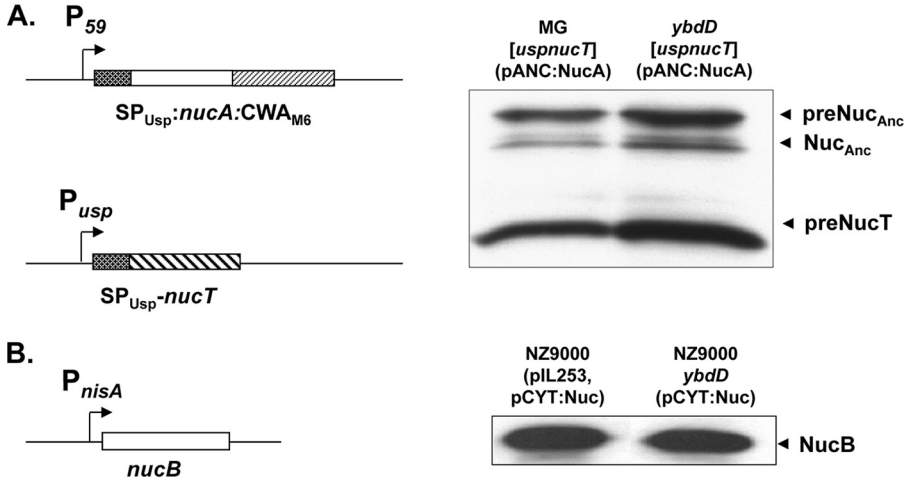

FIG 4 Effect of *ybdD* mutation on differently localized form of Nuc. (Left) Schematic representation of the *nuc* expression cassettes tested. (Right) Protein samples from cell fractions of exponentially growing cultures were analyzed by SDS-PAGE and Western blotting using anti-Nuc antibodies. (A) Coproduction analysis of cell wall-anchored nuclease (Nuc_{Anc}) and pre-NucT in MG[*usp-nucT*](pANC:NucA) and the *ybdD*[*usp-nucT*](pANC:NucA) mutant. (B) Production analysis of a cytoplasmic form of Nuc induced by 1 ng/ml nisin for 1 h in NZ(pIL253, pCYT:Nuc) and NZ*ybdD*(pCYT:Nuc).

which are present in proteins acting in close association with single-strand nucleic acid (1, 8, 18). Northern blot analysis revealed greater amounts of *nucT* and *usp45* transcripts in the *ybdD*[*usp-nucT*] strain than in the parental strain MG[*usp-nucT*], suggesting that YbdD could act at the transcriptional step in protein synthesis process (see Fig. S2 in the supplemental material). Assessment of the mRNA levels of other homologous cytoplasmic (Ldh and HslA) and membrane proteins (PmpA and SipL) did not reveal any increase in the *ybdD* mutant. These observations suggest that *ybdD* could modulate the expression of some specific exported proteins in *L. lactis*, addressing the question of YbdD targeting and indicating that the exportation signal is at the protein level. Further functional and molecular characterization of YbdD should be of particular interest, possibly allowing the identification of a new regulator and/or mechanism involved in the exported-protein synthesis process.

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